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Award Number: DAMD17-00-1-0041

TITLE: Proto-oncogene PML and Tumor Evasion in Prostate Cancer

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REPORT DATE: November 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2000	Annual (1 Nov 99 - 31 Oct 00)

4. TITLE AND SUBTITLE
Proto-oncogene PML and Tumor Evasion in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0041

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The peptides presented by class I human leukocyte antigen (HLA) are the primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). Tumors can therefore avoid CTL recognition by down-regulation of cell surface HLA. However, the molecular mechanism of HLA down-regulation and its significance in progression of prostate carcinoma have not been determined. We have proposed to identify the antigen presentation defects in prostate cancer, to examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer, and to study the immune regulation in experimental transgenic murine prostate cancer (TRAMP) models. In the past funding period, we have performed immunohistochemical study to show the concordant proto-oncogene PML and HLA class I antigen down-regulation in surgically removed prostate cancer lesions. We have examined the proto-oncogene PML isoform expression and antigen presentation gene expression in prostate cancer cell lines. Most importantly, we made the important discovery that thymic deletion is the major mechanism of T cell immune tolerance in TRAMP mouse model. We believe that these works will open new revenues for prostate cancer immunotherapy.

14. SUBJECT TERMS
Immune interactions, immunotherapy, oncogene expression, tumor progression.

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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(4) Introduction

The peptides presented by class I human leukocyte antigen (HLA) are the primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). Tumors can therefore avoid CTL recognition by down-regulation of cell surface HLA. Previous study showed that 34% of primary prostate cancer and 80% of metastatic prostate cancer in lymph nodes had total loss of MHC class I expression. However, the molecular mechanism of MHC down-regulation and its significance in progression of prostate carcinoma have not been determined. Cell surface expression of HLA requires coordinated expression of multiple genes, encoding, respectively, proteosome component LMP-2/7, peptide transporter TAP-1/2 , β-2 microglobulin, and class I heavy chains. While inactivation of either one of these genes is sufficient to cause severe antigen-presentation defects, expression of all of these genes are simultaneously repressed in many types of tumor. The coordinated expression and repression of these genes indicates presence of a master regulator for MHC class I antigen-presentation. We have recently revealed that proto-oncogene PML controls multiple genes devoted to antigen-presentation, and that malfunction of PML leads to down regulation of MHC class I and tumor recurrence in experimental murine tumors. Here we propose to investigate if malfunction of PML is responsible for antigen-presentation defects in prostate cancer, and whether such defects contribute to tumor progression in human and transgenic mouse models. Our proposal has three specific aims. First we will identify the antigen presentation defects in prostate cancer. We will systematically examine the mRNA expression of multiple genes devoted to HLA class I antigen presentation, including HLA class I heavy chain, β_2-microglobulin (β2m), TAP-1, TAP-2, LMP-2 and LMP-7 in radical prostatectomy specimens. Next we will examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer. We will determine whether PML malfunction is responsible for HLA down regulation in prostate cancer samples. Furthermore, we will examine the immune regulation and tumor evasion mechanisms in experimental transgenic murine prostate cancer (TRAMP) models.

(5) Body of Annual Report

Statement of Work:

Task 1. To identify the antigen presentation defects in prostate cancer.

- *Develop the patients' database for 308 cases of radical prostatectomy specimens we collected during the past four years.*
- *Identify the prostate cancer samples that have complete loss of HLA class I expression by performing immunohistochemistry study on the archived formalin fixed paraffin embedded prostate cancer tissue samples with anti-HLA class I antibody HC10 on 308 cases of radical prostatectomy specimens.*
- *Collect the HLA class I positive tumor tissue, HLA class I negative tumor tissue, HLA class I positive normal or hyperplastic prostatic tissue by Laser Capture Microdissection (LCM) on ethanol fixed paraffin embedded tissue sections.*
- *Systematically examine the mRNA expression of multiple genes devoted to MHC class I antigen presentation, including MHC class I heavy chain, β_2M, TAP-1, TAP-2, LMP-2 and LMP-7 by RT-PCR and real time PCR among different groups of tissue.*

Task 2. To examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer.

- *Transfect PML cDNA into MHC class I negative prostate cancer cell line(s) (LnCaP as one example) to investigate whether overexpression of PML complements the antigen presentation defects and upregulates MHC class I expression.*
- *Examine the PML protein expression in prostate cancer by immunohistochemistry study using monoclonal antibody against PML (PG-M3).*
- *Examine the correlation between down-regulation of HLA class I and expression of PML protein by examine the mRNA expression of PML from the tissues being collected by Laser Capture Microdissection (LCM).*
- *Purify the genome DNA from ethanol fixed paraffin embedded tissue section and sequence the PML gene to determine if PML mutation(s) may be responsible for the defective expression of cell surface HLA class I.*

Accomplishment:

During the past funding period, we have made important progress in accomplishing Task 1 and Task 2.We have collaborated with Dr. Jonathan Melamed in the Department of Pathology at New York University Medical Center and Dr. Saldano Ferrone in the Department of Immunology at Roswell Park Cancer Institute. We have examined the concordant expression of proto-oncogene product PML and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions. Immunohistochemical staining of formalin fixed paraffin embedded sections with anti-HLA class I heavy chain monoclonal antibody (mAb) HC-10 detected their down-regulation in 27 lesions (73%), although with a different extent (50-90% of carcinoma cells were not stained by mAb HC-10), which is much higher than previously reported (1). Furthermore immunohistochemical staining with anti-PML mAb PG-M3 showed that 23 of the 27 lesions (85%) with HLA class I antigen downregulation had also down-regulation of PML nuclear expression (17 cases with complete lack of reactivity to PG-M3 and 6 cases with weak reactivity to PG-M3). Morphologically, the

Gleason grade 3C carcinoma that consists of well circumscribed cribriform tumor mass is the most common type that exhibits simultaneous complete loss of HLA class I and PML expression. In summary, our results suggest that PML down-regulation is strongly associated with HLA class I down-regulation in prostate cancer. Our results have been submitted as abstract to Experimental Biology 2001 (Appendix 1).

We have systematically examined the HLA class I and PML isoform expression in various human tumor cell lines, including prostate cancer cell lines (Du145, PC3, LnCap), melanoma cell lines (SK-Mel-19, 1092, 1195), and small cell lung carcinoma cell lines (H146, H1095). We performed Northern Blot experiments to examine the mRNA level for antigen presentation genes, including MHC class I heavy chain, β_2M, TAP-1, TAP-2, LMP-2 and LMP-7, as well as PML isoforms. We have identified seven different isoforms of PML in different splcing forms. Certain isoform of PML appears to correlate with HLA class I expression. We are in a process of subcloning the different isoforms of PML in mammalian expressing vector. We will test whether overexpression of PML may compensate the antigen presentation defects in human tumor cell lines.

Due to the financial restraint, we do not have the manpower to develop the prostate cancer database. We also encountered some difficulty to obtain prostate cancer tissue blocks from other institutes. Fortunately, we recently obtained a new grant from the Cancer Research Institute to establish a new prostate cancer tissue resource in central Ohio area. We now have a research nurse to coordinate the tissue bank work and we expect to establish the prostate cancer tissue bank database in next funding period. The database is expected to include approximately 3000 prostate cancer patients.

We have worked successfully to obtain tissues by Laser Capture Microdissection, isolated DNA and performed real time PCR with LightCycler from the Roche Molecular Biochemicals. However, we encountered some technical difficulties in isolating RNA from the tissue that obtained by Laser Capture Microdissection. We will continue to work on the same direction that outlined in Task 1 and Task 2 in the next funding period.

Statement of Work

Task 3. To test whether overexpression of PML and upregulate MHC class I expression in vivo will improve the overall prognosis in experimental murine prostate cancer model.

- *Obtaining "TRAMP" mice from The Jackson Laboratory.*
- *Examine the cell surface MHC class I expression in prostate cancer samples from TRAMP mice to determine whether down regulation of MHC class I heavy chain, β_2-microglobulin (β_2M), TAP-1, TAP-2, LMP-2 and LMP-7 correlates with progression of murine prostate cancer.*
- *Breed TRAMP mice with β_2M -/- mice to determine whether ablation of MHC class I will accelerate tumor progression.*
- *Make the PML transgenic construct under the control of rat probasin promoter which has tissue specific expression in prostate gland.*
- *Produce the PML transgenic mice that over-express PML in prostate gland.*
- *Breed the PML transgenic mice with TRAMP mice and determine the effect of PML on antigen presentation and tumor progression.*
- *Produce the costimulatory molecule B7-1 transgenic mice under the control of rat probasin promoter.*

- *Breed the B7-1 transgenic mice with TRAMP mice, and furthermore, breed the B7-1, PML transgenic mice with TRAMP mice to examine the effects of B7 or B7 plus MHC class I expression in vivo on the incidence of spontaneous prostate cancer.*

Accomplishment

The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer(2,3). We obtained the TRAMP mice as scheduled and established our own colony. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. We immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) (4) and analyzed the antigen specific T cell response by ELISPOT. We could not detect any antigen specific T cell response to Tag epitope IV in TRAMP mice, which was in consistent with the previous report (5).

We established the collaboration with Dr. T. Geiger from St. Jude Children's Hospital to study the mechanism of immune tolerance in TRAMP mice. Dr. Geiger had made a TCR transgenic mouse model TG-B mice that is transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-$2K^k$ (6,7). We have further mapped the SV40 Tag epitope to peptide 560-568. The transgenic T cells from TG-B mice respond vigorously to this nenopeptide both in proliferation assay and in cytotoxic killing assay. The T cells respond to $1x10^{-5}$ μg/ml peptide concentration instead of 20 μg/ml of original 18-aa peptide in published literature in proliferation assay (8).

To examine whether the immune tolerance is due to thymic deletion, we crossed the TRAMP mice with TG-B mice. Double transgenic TRAMP/TCR mice had thymic deletion of SV40 Tag reactive T cells when examined at 25 days after birth. The thymus size is reduced from $6.5x10^7$ thymocytes in TCR transgenic mouse to $5x10^6$ thymocytes in double transgenic mouse. The mature CD8+Vb8+ T cells from spleen are reduced from $5x10^6$ cells in TCR transgenic mouse to $1x10^5$ cells in double transgenic mouse that possibly represent the endogenously rearranged Vb8+ T cells. The thymic deletion of SV40 Tag specific T cells is identified in both male and female double transgenic TRAMP/TCR mice. We subsequently detected the message for the SV40 Tag in the thymus of the double transgenic TRAMP/TCR mice and TRAMP mice through RT-PCR and Southern blot. Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice. The study is submitted as an abstract to EB2001 (Appendix 2).

In addition to above study, we have made several transgenic mice lines that outlined in Statement of Work. First is the transgenic mouse that has proto-oncogene PML under the control of rat probasin promoter. The second is the transgenic mouse that has co-stimulatory molecule B7-1 under the control of rat probasin promoter. We are currently in process of further analyzing the transgene expression pattern and in crossing the mice to TRAMP mice.

(6) Key Research Accomplishments

- We have examined the concordant expression of proto-oncogene product PML and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions.
- We have systematically examined the HLA class I and PML isoform expression in various human tumor cell lines, including prostate cancer cell lines. We performed Northern Blot experiments to examine the mRNA level for antigen presentation genes, including MHC class I heavy chain, β_2M, TAP-1, TAP-2, LMP-2 and LMP-7, as well as PML isoforms.
- We obtained the TRAMP mice as scheduled and established our own TRAMP mice colony in our animal facility.
- We immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) that is presented by MHC class I molecule H-2 K^b and analyzed the antigen specific T cell response by ELISPOT. We confirmed the previous observation that T cells are immune tolerant in TRAMP mice.
- We have obtained a TCR transgenic mouse model TG-B mice that is transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-2K^k.
- We have further mapped the SV40 Tag epitope to peptide 560-568. The transgenic T cells from TG-B mice respond vigorously to this nenopeptide both in proliferation assay and in cytotoxic killing assay.
- We have crossed the TRAMP mice with TG-B mice. The analysis showed complete thymic deletion of SV40 Tag reactive T cells occurred in the double transgenic mice.
- We have shown that SV40 Tag mRNA can be detected in thymus and spleen by RT-PCR and ECL Southern Blot in TRAMP mice and in TRAMP/TG-B double transgenic mice.
- Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice.
- We have produced the transgenic mouse that has proto-oncogene PML under the control of rat probasin promoter, and the transgenic mouse that has co-stimulatory molecule B7-1 under the control of rat probasin promoter.

(7) Reportable Outcomes:

- Experimental Biology 2001 abstract #4092: Thymic deletion of specific T cells reactive to SV40 large T antigen in TRAMP mice.
- Experimental Biology 2001 abstract #4210: Concordant proto-oncogene PML and HLA class I down-regulation in surgically removed prostate cancer lesions: an immunohistochmical study.
- New funding:
    - Cancer Research Institute, Prostate Cancer Initiative, Pre-clinical Award, "Prostate Cancer Tissue Resource", Principal Investigator: Pan Zheng, MD, PhD, 09/01/00-08/31/03, annual direct cost: $150,000/yr.

(8) Conclusions:

In summary, we have made important progress during the first funding period to achieve our goal. We have followed the Statement of Work closely. We have established the concordant proto-oncogene PML and HLA class I antigen down-regulation by immunohistochemical study. We have examined the proto-oncogene PML isoform expression and antigen presentation gene expression in prostate cancer cell lines. Most importantly, we made the important discovery that thymic deletion is the major mechanism of T cell immune tolerance in TRAMP mouse model.

Our progress is fundamentally important for future development of new ways for immunotherapy and tumor vaccines. Our collaborator, Dr. Yang Liu from Department of Pathology in The Ohio State University, has recently discovered that peri-natal administration of anti-B7 antibodies altered the T cell repertoire during the T cell development (9). Our preliminary study indicated that anti-B7 antibody treatment to adult mice might also change the T cell repertoire to induce the development of SV40 Tag reactive T cells in TRAMP/TG-B double transgenic mice. We will follow the observation to test whether we might break the immune tolerance in TRAMP mice using anti-B7 treatment.

(9) References:

1. Blades, R.A., P.J. Keating, L.J. McWilliam, N.J. George, and P.L. Stern. 1995. Loss of HLA class I expression in prostate cancer: implications for immunotherapy. *Urology* 46:681-686; discussion 686-687.
2. Greenberg, N.M., F. DeMayo, M.J. Finegold, D. Medina, W.D. Tilley, J.O. Aspinall, G.R. Cunha, A.A. Donjacour, R.J. Matusik, and J.M. Rosen. 1995. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 92:3439-3443.
3. Gingrich, J.R., R.J. Barrios, R.A. Morton, B.F. Boyce, F.J. DeMayo, M.J. Finegold, R. Angelopoulou, J.M. Rosen, and N.M. Greenberg. 1996. Metastatic prostate cancer in a transgenic mouse. *Cancer Res* 56:4096-4102.
4. Mylin, L.M, R.H. Bonneau, J.D. Lippolis, and S.S. Tevethia. 1995. Hierarchy among multiple H-2b-restricted cytotoxic T-lymphocyte epitopes within Simian Virus 40 T antigen. *J Virol.* 69:6665-6677.
5. Granziero L, S. Krajewski, P Farness, L Yuan, M.K. Courtney, MR Jackson, PA Peterson, and A. Vitiello. 1999. Adoptive immunotherapy prevents prostate cancer in a transgenic animal model. *Eur J Immunol* 29:1127-1138.
6. Geiger T, LR Gooding, and RA Flavell. 1992. T-cell responsiveness to an oncogenic peripheral protein and spontaneous autoimmunity in transgenic mice. *Proc Natl Acad Sci U S A.* 89:2985-2989.
7. Soldevila G, T Geiger, and RA Flavell. 1995. Breaking immunologic ignorance to an antigenic peptide of simian virus 40 large T antigen. *J Immunol* 155:5590-5600.
8. Rawle FC, KA O'Connell, RW Geib, B Roberts, and LR Gooding. 1988. Fine mapping of an H-2Kk restricted cytotoxic T lymphocyte epitope in SV40 T antigen by using in-frame deletion mutants and a synthetic peptide. *J Immunol* 141:2734-9.
9. Gao JX, H. Zhang, XF Bai, J Wen, JQ Liu, P Zheng, and Y. Liu. 2000. Induction of multi-organ autoimmune diseases by perinatal blockade of T cell tolerance in the thymus. (submitted).

Appendix 1.

Concordant Proto-oncogene PML and HLA Class I Down-regulation in Surgically Removed Prostate Cancer Lesions: An Immunohistochemical Study

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Antigen peptides presented by the class I major histocompatibility complex (MHC) molecules are primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes(CTL). As a result MHC class I down-regulation which is frequently found in malignant tumors has a negative impact on their recognition by T cells. Normal cell surface MHC class I expression requires coordinated expression of multiple genes encoding, respectively, proteosome components LMP2/7, peptide transporter TAP1/2, β_2 microglobulin(β_2M) and MHC class I heavy chain. We have previously reported that proto-oncogene product PML induces expression of TAP1, TAP2, LMP2 and LMP7 in an MHC class I negative, recurrent tumor, leading to the re-expression of cell surface MHC class I in tumors and to rejection of tumors (Nature, 396:373-376). In this study, we examined the expression of proto-oncogene product PML expression and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions. Immunohistochemical staining of formalin fixed paraffin embedded sections with anti-HLA class I heavy chain monoclonal antibody (mAb)HC10 detected their down-regulation in 27 lesions (73%)with different extent (50-90% of carcinoma cells were not stained by mAb HC10). Furthermore immunohistochemical staining with anti-PML mAb PG-M3 showed that 23 of the 27 lesions (85%) with HLA class I antigen downregulation had also down-regulation of PML nuclear expression (17 cases with complete lack of reactivity to PG-M3 and 6 cases with weak reactivity to PG-M3). Morphologically, the Gleason grade 3C carcinoma that consists of well circumscribed cribriform tumor mass is the most common type that exhibits simultaneous complete loss of the HLA class I and PML expression. In summary, our results suggest that PML down-regulation is strongly associated with HLA class I down-regulation in prostate cancer.

Appendix 2.

Thymic Deletion of Specific T Cells Reactive to SV40 Large T Antigen in TRAMP Mice

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The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. In this study, we immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) and analyzed the antigen specific T cell response by ELISPOT. We could not detect any antigen specific T cell response to Tag epitope IV in TRAMP mice, which was in consistent with the previous report. To examine whether the immune tolerance is due to thymic deletion, we crossed the TRAMP mice with TG-B mice transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-2K^k. Double transgenic TRAMP/TCR mice had thymic deletion of SV40 Tag reactive T cells when examined at 25 days after birth. The thymus size is reduced from $6.5x10^7$ thymocytes in TCR transgenic mouse to $5x10^6$ thymocytes in double transgenic mouse. The mature CD8+Vb8+ T cells from spleen are reduced from $5x10^6$ cells in TCR transgenic mouse to $1x10^5$ cells in double transgenic mouse that possibly represent the endogenously rearranged Vb8+ T cells. The thymic deletion of SV40 Tag specific T cells is identified in both male and female double transgenic TRAMP/TCR mice. We subsequently detected the message for the SV40 Tag in the thymus of the double transgenic TRAMP/TCR mice and TRAMP mice through RT-PCR and Southern blot. Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice.